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ORIGINAL PAPER

EVALUATION OF THE TIME STABILITY OF AORTIC RINGS IN YOUNG WISTAR RATS DURING AN EIGHT-HOUR INCUBATION PERIOD*

Michał Majewski[1], Małgorzata Lepczyńska[2], Ewa Dzika[2], Waldemar Grzegorzewski[1], Włodzimierz Markiewicz[3], Marta Mendel[4], Magdalena Chłopecka[4]

[1] Department of Pharmacology and Toxicology
[2] Department of Medical Biology
[3] Department of Pharmacology and Toxicology
University of Warmia and Mazury in Olsztyn, Poland
[4] Department of Preclinical Sciences
Warsaw University of Life Sciences – SGGW, Poland

Abstract

Vascular disorders are a major problem as their pathophysiology has not been fully understood, therefore animal models are implemented to extrapolate the results obtained on animals to humans. The isolated tissue bath technique is a classical pharmacological tool for evaluating concentration-response relationship and to measure the isometric contraction of isolated large conduit (thoracic aorta) arteries. The aim of this study was to investigate the stability of thoracic rings from 10-week-old male Wistar rats during 2, 4, 6 and 8 h of incubation. The vascular response to potassium chloride (KCl), noradrenaline (NA) and acetylcholine (ACh) was analyzed in tissue baths. In addition, histological morphology of the aortic rings after the incubation period was studied. No difference was observed in the contraction to NA (mg of tension, 8 h: 1711 ± 53.85 *vs.* control: 1567 ± 48.55, $P>0.0928$) and vasodilation to ACh (% relaxation, 8 h: 64.7 ± 4.05 *vs.* control: 71.21 ± 3.613, $P>0.05$) of the thoracic rings among the investigated groups of rats. The integrity of the tunica media and adventitia structure remained unaffected during the first 6 h of incubation. Within 8-hour incubation, slight degeneration of the tunica media smooth muscle structure took place. The results indicate that incubation carried up to 6 h constitutes a reliable experimental model, as to the NA and ACh application. However, longer experiments should be performed with caution due to the appearance of some initial structural changes of the tunica media smooth muscle cells of a rat aorta starting from the eighth hour of incubation. Thus, it is reasonable to carry out similar experiments with an extended range of experimental reagents, and in different pathological conditions to verify the results obtained in this experiment.

Keywords: aorta, aortic rings, concentration-response curve, incubation, isometric contraction, smooth muscle.

Michał Majewski, PharmD, Department of Pharmacology and Toxicology, Centre of Experimental Medicine, Warszawska 30, 10-082 Olsztyn, Poland, e-mail: michal.majewski@uwm.edu.pl
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INTRODUCTION

Studies on isolated blood vessels are crucial for the examination of the development and safety of drugs with potential regulatory mechanisms, as well as for functional and biomechanical investigations (Neves et al. 2012, Jiang et al. 2014, Karpińska et al. 2017). Concentration-response curves of both aorta and mesenteric vessels are frequently used in the studies of obesity (Majewski et al. 2018), aging (Ibarra et al. 2006), hormonal imbalance (Zaki, Youssef 2013, Isidoro et al. 2018) and exercise training (Han et al. 2018). Rat arteries are also studied in many different types of dietary conditions with either implementation or deficiency of nutrients, such as copper (Majewski et al. 2017), omega-3 fatty acids (Villalpando et al. 2017), resveratrol (Han et al. 2018), or in chronic alcoholism (Cerqueira et al. 2005). The most common forms of aortic disease are aneurism, diabetes, hypertension, obesity, atherosclerotic occlusion and age-induced vascular stiffening (Cattell et al. 1993, Török, Kristek 2001, Lindeman et al. 2010, Nguy et al. 2012, Podzolkov et al. 2014, Majewski et al. 2018). These conditions are currently of a serious health concern because they are common and can lead to fatal outcomes.

Experiments on thoracic arteries flourished in the mid-20th century, and the versatility, simplicity and reproducibility of such assays make them an indispensable tool for pharmacologists and physiologists alike (Jespersen et al. 2015).

However, we have not found any study that has evaluated the isometric force and histological status of aortic preparations from young Wistar rats during 8-hour incubation. Hence, the aim of this study was (i) to evaluate the time-stability of rat aortic rings treated with potassium chloride (KCl), noradrenaline (NA) and acetylcholine (ACh), and (ii) to examine the potential aortic structure in the analyzed aortic rings.

MATERIAL AND METHODS

This study was performed in accordance with the European guidelines (Directive 2010/63/EU for animal experiments) and conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 86–26, revised 2014).

Animals

Eight healthy male albino Wistar rats (10-week-old, having the body weight of 300-350 g) were subjected to the experiment. The animals were kept under laboratory conditions of a 12-h light-dark cycle, temp. of 21-22°C, ventilation rate of 20 air changes per hour and relative humidity 50 ± 10%. Water and standard rat feed were supplied *ad libitum*.

Drugs and reagents

The drugs used were: acetylcholine chloride, noradrenaline hydrochloride, potassium chloride (Sigma-Aldrich). Stock solutions (10 mM) were prepared in distilled water. The solutions were maintained at –20°C and appropriate dilutions were made in Krebs-Henseleit solution (KHS) on the day of the experiment.

Preparations of aortic rings and isometric vascular tone recording

The rats were euthanized and decapitated. Immediately, the thoracic cavity was opened through a median sternotomy and the thoracic aorta was excised carefully to avoid damage to the aortic wall, after which it was placed into ice-cold KHS (in mM: 118,4, NaCl; 4.7, KCl; 1.2, $MgSO_4$; 2.5, $CaCl_2$; 1.2, KH_2PO_4; 25, $NaHCO_3$; 11.1, glucose), aerated with a mixture of 95% oxygen and 5% carbon dioxide. The vessels were cleaned of connective tissue and cut into 8 rings (3-4 mm in length). The effects of changes in the vascular tone of isolated aortic rings were determined in organ baths containing 5 ml KHS. The solution was aerated, and maintained at 37°C. The rings were horizontally mounted between two stainless hooks.

Selection of loading and the reference dose

We chose the NA concentration at 0.1 μM and the resting tension at 1 g, based on results obtained in the preliminary experiments. NA used at this concentration allowed the constriction of the aortic rings with a submaximal concentration of 80% (~EC80 level).

Experimental design

The rings, maintained in KHS at 37°C and pH 7.4, were subjected to a resting loading tension of 1 g which was readjusted every 15 min during a 60 min equilibration period before further examination. Then, vessels were exposed, for the first time, to KCl (75 mM) – control. The rings were rinsed with KHS and after the application of the required tension, the preparations were allowed to equilibrate for 2, 4, 6 or 8 h in KHS. The contractile responses elicited by a second single depolarizing concentration of KCl (75 mM) were assessed to examine possible changes in the functional integrity. After washing in KHS, the cumulative concentration-response to NA (0.1 nM-10 μM) was performed at 2, 4, 6 or 8 h. In another set of experiments, the cumulative concentration-response curve to ACh (0.1 nM-10 μM) was obtained in NA precontracted rings. The presence of vascular endothelium was confirmed by the ability of ACh (10 μM) to relax rings precontracted with NA (0.1 μM).

The histopathological study of the rat aorta

After 2, 4, 6 and 8 h of incubation, the aortic rings were removed from the organ baths (without any chemical stimuli), fixed in 10% neutral buffered formalin and embedded in paraffin. The solidified tissues were sectioned at a 5 μm thickness using a microtome. A minimum of 4 to 8 sections

were prepared from each specimen. The sections were stained with haematoxylin and eosin and underwent histological examination. The artery rings obtained from the rats immediately after euthanasia served as a control. Images were captured using an Olympus BX41 optical microscope connected to a computer with a high-resolution screen, where the images were processed and digitized.

Statistical analysis

Graphs were built and analyzed in GraphPad Prism 7. Contraction was expressed in mg of developed tension for both KCl and NA. Vasodilation was represented as a percentage of the maximal response to NA (0.1 μM). Data are expressed as the means of 8 independent experiments ± S.E.M (Standard Error of the Mean), between the studied groups at 2, 4, 6 and 8 h of incubation and were compared by ANOVA with Tukey's multiple comparisons test, where appropriate. The model assumption of normality and homogeneity of variance was tested. A value of $P \leq 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Vascular reactivity studies

The contractile response generated by a single dose of 75 mM KCl was not significantly different between the analyzed groups (mg) 2-hour: 1512 ± 94.26, 4-hour: 1769 ± 45.16, 6-hour: 1786 ± 69.67 and 8-hour: 1468 ± 107.2, and with the KCl control: 1567 ± 48.55 (all values of $P > 0.0928$ ANOVA) – Figure 1.

No difference was observed in the maximal isometric contraction of the thoracic rings subjected to cumulative concentrations of NA (0.1 nM-10 μM) among the investigated groups of rats, as presented in Figure 1 and in Table 1.

The vasodilator response induced by ACh (0.1 nM-10 μM) was similar in aortic rings from all rats ($P > 0.05$ ANOVA) – Table 1 and Figure 2.

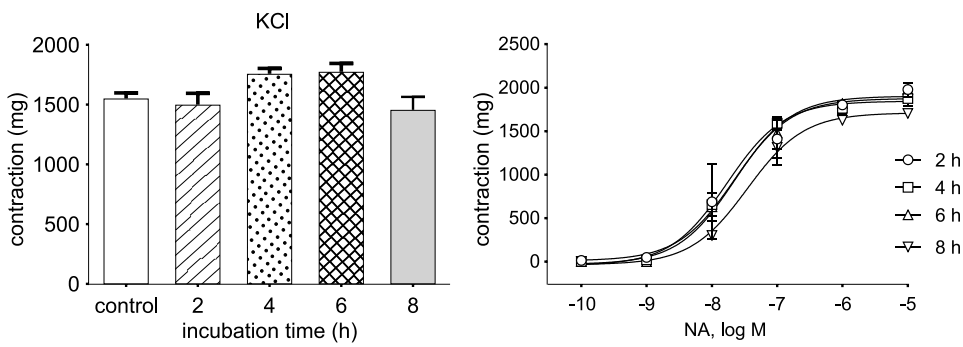

Fig. 1. The concentration-response to potassium chloride (KCl, 75 mM) and to noradrenaline (NA, 0.1 nM-10 μM) during 2, 4, 6 and 8 h of incubation. Control KCl was obtained after 60 min of equilibration period. Results (mean ± SEM, $n = 8$) are expressed in mg of tension, $P > 0.05$ (ANOVA/Tukey's

Table 1

The influence of the incubation time on E_{max} and pD_2 values in noradrenaline (0.1 nM-10 μM) and acetylcholine (0.1 nM-10 μM) induced responses

Incubation time (h)	Parameter	Noradrenaline (mg)	Acetylcholine (%)
2	E_{max}	1876 ± 123.9	71.21 ± 3.613
	pD_2	7.654 ± 0.209	7.145 ± 0.143
4	E_{max}	1845 ± 47.04	73.88 ± 4.738
	pD_2	7.77 ± 0.079	7.002 ± 0.173
6	E_{max}	1903 ± 66.17	72.2 ± 2.845
	pD_2	7.65 ± 0.108	6.891 ± 0.093
8	E_{max}	1711 ± 53.85	64.7 ± 4.05
	pD_2	7.46 ± 0.093	6.958 ± 0.151

Values are expressed as mean ± SEM, $n = 8$, $P>0.05$ (ANOVA/Tukey's);
E_{max}, maximal effect; pD_2, drug concentration exhibiting 50% of the E_{max} expressed as negative log molar

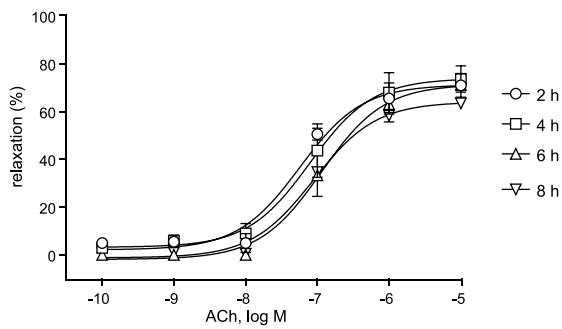

Fig. 2. The cumulative concentration-response curves to acetylcholine (ACh, 0.1 nM–10 μM) during 2, 4, 6 and 8 h of incubation. Results (mean ± SEM, $n = 8$) are expressed as a percentage of inhibition of the contraction induced by NA (0.1 μM), $P > 0.05$ (ANOVA/Tukey's

Histological evaluation of the thoracic aortic structures

For years, the microstructure of the aortic tissue has been studied with great interest. An alteration of the quantity and/or architecture of the connective fibers, as well as the expression and localization of different receptors (adrenergic) within the aortic wall and nitric oxide (NO) bioavailability may directly impart elasticity, strength and functioning, which leads to mechanical and functional alterations associated with pathophysiological conditions (Sokolis et al. 2010, Tsamis et al. 2013, Majewski et al. 2017, Rodríguez et al. 2017, Majewski et al. 2018).

In numerous studies, the experiments on isolated tissues have been commonly performed on strips or rings as well as on isolated perfused blood vessel segments (Chłopecka et al. 2007, Assoul et al. 2008, Neves et al. 2012, Majewski et al. 2018). A great number of studies were carried out, testifying

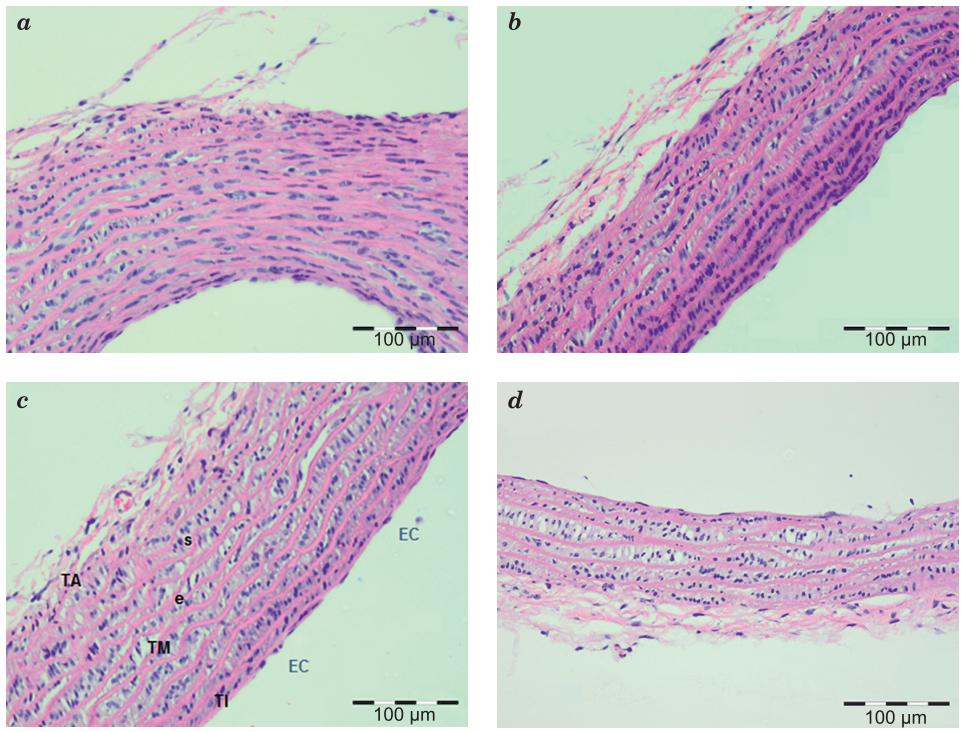

Fig. 3. Representative histological longitudinal sections of the thoracic aorta stained with hematoxylin and eosin (×20) and incubated for (*a*) 2, (*b*) 4, (*c*) 6 and (*d*) 8 h in organ baths (without any chemical stimuli). The aortic rings underwent histological examination. Endothelial cell (EC) line of the tunica intima (TI), elastic lamellae (e), and smooth muscle cell nuclei (s) of the tunica media (TM), and the tunica adventitia (TA)

to the fact that the aorta possesses a non-uniform structure displaying distinct regions that are more susceptible to certain types of disease than other organs (Cattell et al. 1993, Lindeman et al. 2010, Podzolkov et al. 2014). This is the reason why these experiments became the basis for all further studies on vasculature. The integrity of the vascular smooth muscle layer is crucial in maintaining normal vascular morphology and tone (Leone, Coelho 2004, Koole et al. 2013, Serhatli et al. 2014), and changes in the arterial wall composition, with the expression or localization of α_1-adrenergic receptors and integrity of endothelium, may alter its function and lead to the development of vascular disease observed in obesity and diabetes (Majewski et al. 2018) and hypertension (Buchwalow et al. 2008, Rodríguez et al. 2017).

Artery structure varies according to the selected region and physiological state. The thickness of the tunica media reduces its parameters along its length from the proximal end to the distal end (Kneubil et al. 2006) and this increases with age, from the moment of birth to the adult stage (Nowak et al. 2011). Thus, tunica media in young Wistar rats will be thinner than in adult

animals. Also, both expression and localization of the adrenergic receptors in the aorta may vary. Adrenergic receptors (α_{1A} and α_{1D}) have a higher density in the tunica intima and tunica media of spontaneously hypertensive rats compared with normotensive rats (Rodríguez et al. 2017).

After binding to the muscarinic receptors, acetylcholine induces the release of (i) NO through the endothelial nitric oxide synthase and (ii) prostanoids through the cyclooxygenase pathway. In addition, NO bioavailability has been described to be influenced by prostanoids (Majewski et al. 2018), however hyperpolarizing mechanisms are able to counterbalance the predominance of vasoconstrictor prostanoids derived from cyclooxygenase-2, which may account for the maintenance of the ACh-induced relaxation in aortas from experimental rats.

We observed no morphological changes up to the 6th hour of the experiment (Figure 3). The tunica intima was still composed of continuous layers of endothelial cells. The tunica media appeared to have numerous normal and healthy distinct elastic laminae, which were wavy and arranged concentrically, with smooth muscle cells seen in the interspaces between the concentric lamellae. There were no significant changes in the thickness of the tunica media. The tunica media was recognized as being comprised of elastic membrane layers between which there were the smooth muscle cells and a small amount of collagen and elastic fibers. The tunica adventitia was recognized by the normal-looking fibrous tissue elements.

Starting at the 8th hour of incubation, only a very few rings of the rat aorta revealed some morphological alterations. The observed alterations were limited to the tunica media, which showed slight degeneration and necrosis of the smooth muscle cell nuclei.

It is worth emphasizing the relation between the apparent changes in the artery's morphology and the aortic rings' reactivity to the non-selective α-adrenergic receptors agonist, NA, throughout the entire incubation period. The central role of α_1-adrenergic (α_{1A} and α_{1D}) receptors is to regulate vascular tone and accordingly blood pressure, in addition to which it regulates vasoconstriction and, to a lesser extent, cardiac contractility. The integrity of the tunica media and adventitia structure remained unaffected during the first 6 h of incubation. Within the 8 h of the experiment, slight degeneration of the tunica media smooth muscle structure took place and therefore the NA induced contraction was slightly attenuated compared to the results obtained at the beginning of the experiment. As α_1-adrenergic receptors are also localized in the tunica media, some histological changes which occurred near the end of the experiment may explain the attenuation in NA induced response.

CONCLUSIONS

This *in vitro* model may be characterized by its relative stability, which places it in a good standing as no morphological changes were observed at up to the 6th hour of the experimental treatment.

However, further analysis involving ultrastructural and biochemical evaluation of the aortic wall components are necessary and should be performed on different animals, different age groups, and/or with animal models that simulate various health-related conditions.

Conflict of interests

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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